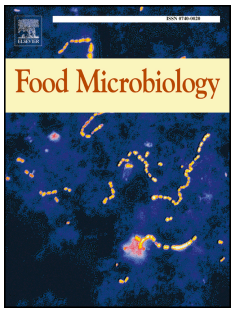

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Stress-resistance associated with multi□host transmission and enhanced biofilm formation at 42ºC among hyper-aerotolerant generalist *Campylobacter jejuni*

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Abstract

One of the emerging conundrums of *Campylobacter* food-borne illness is the bacterial ability to survive stressful environmental conditions. We evaluated the heterogeneity among 90 *C. jejuni* and 21 *C. coli* isolates from different sources in Egypt with respect to biofilm formation capabilities (under microaerobic and aerobic atmosphere) and resistance to a range of stressors encountered along the food chain (aerobic stress, refrigeration, freeze-thaw, heat, peracetic acid, and osmotic stress). High prevalence (63%) of hyper-aerotolerant (HAT) isolates was observed, exhibiting also a significantly high tolerance to heat, osmotic stress, refrigeration, and freeze-thaw stress, coupled with high biofilm formation ability which was clearly enhanced under aerobic conditions, suggesting a potential link between stress adaptation and biofilm formation. Most HAT multi-stress resistant and strong biofilm producing *C. jejuni* isolates belonged to host generalist clonal complexes (ST-21, ST-45, ST-48 and ST-206). These findings highlight the potential role of oxidative stress response systems in providing cross-protection (resistance to other multiple stress conditions) and enhancing biofilm formation in *Campylobacter* and suggest that selective pressures encountered in hostile environments have shaped the epidemiology of *C. jejuni* in Egypt by selecting the transmission of highly adapted isolates, thus promoting the colonization of multiple host species by important disease□causing lineages.

Keywords: *Campylobacter*, aerotolerance, multi-stress tolerance, generalist lineages, biofilm formation.

1. Introduction

Campylobacter is the most common causative agent of bacterial gastroenteritis worldwide, with particularly high incidence in low- and middle-income countries (Ruiz-Palacios, 2007; Kaakoush *et al.*, 2015). *Campylobacter* is a microaerophilic, fastidious bacterium with an optimal growth temperature range between 37 degrees and 42 degrees Celsius (Garénaux, 2008). *Campylobacter* is part of the intestinal microbiota of a wide range of animals, which can act as reservoirs for zoonotic transmission to humans. Typically, human infection is primarily associated with the consumption of contaminated poultry and meat products, or unpasteurized milk although environmental sources also serve as a transmission route (El-Zamkan and Abdel Hameed, 2016; Newell *et al.*, 2017). Furthermore, transmission of *Campylobacter* can be a result of poor hygiene and/or inadequate food preparation methods in both developed and developing countries (Kennedy *et al.*, 2011; El-Tras *et al.*, 2015). In Egypt, campylobacteriosis is a significant public health burden, causing occasional infection in adults, but more frequently in children under the age of 2 years with an incidence rate of 1.5 episodes per child-year (Rao *et al.*, 2001; ElGendy *et al.*, 2018).

Specific phenotypes appear to be more able to survive and persist under harsh environmental conditions, which favor zoonotic transmission and help combat intervention methods employed in poultry processing plants to decrease *Campylobacter* contamination (Bronowski *et al.*, 2014; Yahara *et al.*, 2017). These include the ability to withstand high oxygen tensions, temperature shifts, high osmolarity and physical treatments with hot water, chilling and freezing, and peracetic acid (PAA) (Chen *et al.*, 2014; Umaraw *et al.*, 2017). Convergent evolution of different survival strategies predates our determination to decontaminate food products, facilitating mechanisms to cope with different stress conditions (Jackson *et al.*, 2009). The ubiquity of *Campylobacter* in the environment challenges our efforts to eliminate this bacterium from the food chain (Omara *et al.*, 2015; Oh *et al.*, 2019), and it is not clear how *Campylobacter* is able to survive the multiple stresses imposed during food preservation, transportation and cooking (Jong *et al.*, 2012). The ability of this bacterium to persist and cause gastroenteritis is in contrast to the difficulty of handling it in the laboratory due to its fastidious nature (Garénaux, 2008; Pascoe *et al.*, 2019).

Nevertheless, the formation of biofilms and the capability to withstand oxidative stress are among the major strategies used by *Campylobacter* to survive under stressful conditions (Pascoe *et al.*, 2015; Karki *et al.*, 2018). Generally, biofilms are defined as multicellular layers of bacteria embedded within a matrix of extracellular polymeric substances that consisted of proteins including enzymes, DNA, RNA, polysaccharides, and water. Bacterial bio-

film is considered as a key player in the bacterial survival in diverse ecological niches (Steenackers *et al*., 2016). Biofilm formation protects bacteria from diverse environmental stressors (Chang *et al.*, 2007). Moreover, bacteria encased in biofilms have been reported to be 1,000-fold more resistant to antimicrobial agents than their planktonic counterparts (Fux *et al.*, 2005), whereas, in human infection, bacteria that produce biofilms are better protected against host defense mechanisms (Ciofu *et al*., 2015). In food production environments, the presence of *Campylobacter* encased in biofilms formed on food processing surfaces protects it from cleaning and sanitation measures, and facilitates dissemination leading to further contamination of various food products, thus increase its potential to cause disease (Nguyen *et al.*, 2012; Yahara *et al*., 2017).

Characterization of *C. jejuni* genotypes based on multi-locus sequence typing (MLST) from the large, curated online pubMLST database[1] provides evidence of host restricted lineages, or host specialists, that are predominantly found in only one particular host species (Dingle *et al.*, 2001; Jolley *et al.*, 2018). On the contrary, host-generalist lineages are more promiscuous and can regularly be isolated from multiple host sources, including the globally disseminated clonal complexes (CCs) ST-21, ST-48, ST-206 and ST-45 (Sheppard *et al.*, 2010, 2014, 2018). Lineages with broad host ranges are frequently isolated from multiple animal species and are a major cause of human disease (Sheppard *et al.*, 2009a, b; Cody *et al.*, 2013). Presumably, host generalist lineages may be better equipped to withstand hostile environmental conditions, but this remains uncharacterized.

To fill this important knowledge gap, the survival rates of 111 *Campylobacter* isolates from different sources in Egypt were screened under different stress conditions which mimic ecological niches encountered in farms and food processing industries and which must be overcome for zoonotic transmission to humans.

2. Materials and Methods

2.1. Bacterial isolates and culture conditions

A total of 111 *Campylobacter* isolates were collected in Cairo, Egypt, from September 2017 to December 2018, including 57 clinical isolates, 24 from dairy products and 30 from broiler carcasses (Supplementary Table S1). Clinical isolates were isolated from stool samples of patients having gastroenteritis admitted to two different hospitals in downtown Cairo. A stratified randomized sampling approach was conducted to include *Campylobacter* isolated from food samples from different retail stores located around the study region. The

isolation and enumeration of *Campylobacter* isolates from different food matrices was performed according to the ISO 10272-1 (Enrichment Method; Detection of *Campylobacter* spp. after Selective Enrichment). All isolates were subcultured from −80°C frozen stocks onto Mueller-Hinton (MH) agar (Oxoid, United Kingdom). Plates were incubated at 42 ±1°C under microaerophilic conditions using AnaeroGen™ 2.5L sachets (Oxoid, United Kingdom). Genomic DNA was extracted from cultures using the QIAamp DNA Mini Kit (QIAGEN, UK), according to the manufacturer's instructions. DNA was quantified using a Nanodrop spectrophotometer before subsequent genome sequencing.

2.2. Genome sequencing

Campylobacter isolates (n=111) were sequenced using an Illumina MiSeq benchtop sequencer (Supplementary Table S2 for genome assembly quality features). Libraries were prepared using the Nextera XT Library Preparation Kit according to standard protocols and sequenced using a 2 × 300 bp paired end v3 reagent kit (Illumina), following the manufacturer's protocol (Kirk *et al.*, 2018). Raw sequence reads are available on the NCBI and the SRA.under.BioProject.PRJNA576513(https://www.ncbi.nlm.nih.gov/bioproject/PRJNA576513). De novo assembly of genomes were done using SPAdes (version 3.8.0) (Bankevich *et al.*, 2012) and archived on the BIGSdb web-based database platform (Jolley and Maiden, 2010), enabling species identification and MLST. *Campylobacter* MLST genotypes (sequence types (STs) were assigned based on allele definitions from pubMLST[1] and CCs were defined by the most common sequence type sharing at least five out of seven alleles (Dingle *et al.*, 2001).

Phylogenetic trees were reconstructed for the 90 *C. jejuni* and 21 *C. coli*. Core genome MLST (cgMLST) analysis was performed using Genome Profiler software (GeP v1.0.1) (Zhang *et al.*, 2015), using *C_jejuni*_NCTC11168 as reference genome for both *C. jejuni* and *C. coli* analysis (Parkhill *et al.*, 2000). Core genome polymorphic genes (*i.e.* those genes with at least one nucleotide difference among all 90 *C. jejuni* or 21 *C. coli* isolates) were selected for cgMLST phylogenetic tree construction and concatenated in a new fasta file using the *extract_concat_cgMLST_genes.rb* ruby script (https://github.com/JoseCoboDiaz/concat_cgMLST_genes). The concatenated gene-by-gene alignments and phylogenetic tree were obtained with MAFFT version 7 (Katoh *et al.*, 2019) using default parameters for alignment and the Neighbor-Joining method, the Jukes-Cantor substitution model and 1000 bootstrap resampling for the construction of the phylogenetic tree. Plots were plotted in using the R-package *ape* to read Newick files.

2.3. Stress tolerance of *Campylobacter* isolates.

For all the stress tolerance tests, *Campylobacter* isolates were grown on MH agar overnight at 42°C under microaerobic conditions generated using the CampyGen system (CN0035, Oxoid). Afterwards, isolates were harvested and resuspended in fresh MH broth to an optical density at 600 nm (OD_{600}) of 0.1 ($\approx 10^8$ colony forming units (CFU) prior to testing their survival to different stressors as described below. Absence of *Campylobacter* in the artificially inoculated chicken skin and milk was assayed by suspension of one gram chicken skin pieces or one ml of milk in 9 ml Bolton selective enrichment broth (Oxoid, United Kingdom), followed by incubation under microaerobic conditions at 41. 5℃ for 48 hours. Afterwards, 100 µL culture was spread on modified charcoal-cefoperazone-deoxycholate agar (mCCDA; Oxoid, United Kingdom) and incubated at 41. 5℃ for 48 hours. All assays included negative control. For all stress tolerance assays, each experiment was performed using three biological replicates with the difference in viable cell counts between replicates was less than 0.5 $\log_{10}$ CFU/mL, all stress tolerance assays results are elucidated in Supplementary Table S1.

2.3.1. Aerotolerance assay

The aerotolerance assay was performed as previously described (Oh *et al.*, 2015). Bacterial suspensions adjusted at OD_{600} of 0.1 were incubated aerobically with shaking at 200 rpm and 42°C. Aliquots from all bacterial suspensions were taken at 0, 12, and 24 h for serial dilution followed by plating onto Preston *Campylobacter* selective agar (Oxoid, United Kingdom) for bacterial enumeration. *Campylobacter* isolates that were not able to survive under aerobic shaking at 200 rpm for 12 h were classified as aero-sensitive (AS), while those that did survive under aerobic shaking at 200 rpm for 12 h were classified as aero-tolerant (AT), and those that survived for more than 24 h under aerobic shaking at 200 rpm were classified as hyper-aerotolerant (HAT). For comparison purposes, a similar experimental design was performed under microaerophilic conditions.

2.3.2. Survival under refrigerated temperature stress

Survival under refrigeration temperature at 4°C was evaluated as previously described (Oh *et al.*, 2017). In brief, small pieces of raw chicken skin (0.2 gram/piece) that were previously prepared by cutting from chicken thighs using a sterile blade, were placed in 96-well microtiter plate. A total of 100 µL of bacterial suspensions

previously adjusted to OD_{600} of 0.1 were used to contaminate the chicken skin samples, followed by incubation of 96-well plates at 4°C. Chicken skin samples were then taken at 0, 1, 3 and 7 days of incubation and transferred to 15 mL falcon tubes containing 1 mL of fresh MH broth, followed by vortexing for 2 minutes and plating of serial dilutions onto Preston *Campylobacter* selective agar for bacterial enumeration.

2.3.3. Survival under chemical decontamination stress

Peracetic acid was used to study the survival rates of *Campylobacter* following a chemical decontamination process, as described (Oh *et al.*, 2018). Raw chicken skin pieces prepared as described earlier were spiked with 100 µL of bacterial suspensions adjusted to an OD_{600} of 0.1 followed by incubation at 4°C for 1 h under microaerobic conditions. Each chicken skin sample was immersed in 750 ppm PAA (Sigma Aldrich, St. Louis, MO, USA) for 15 seconds, washed in ultra-pure water and transferred to 15 mL falcon tubes containing 1 mL of fresh MH broth followed by vortexing for 2 minutes and plating of serial dilutions onto Preston *Campylobacter* selective agar for bacterial enumeration.

2.3.4. Survival following freeze-thaw stress

Following the contamination of each chicken skin piece placed into the wells of 96-well microtiter plates with 100 µL of bacterial suspensions adjusted to an OD_{600} of 0.1, samples were subjected to freezing at –20°C for 1, 3 and 7 days followed by thawing at 4°C for 2 h. The treated chicken samples were subsequently transferred to 15 mL falcon tubes containing 1 mL of fresh MH broth followed by vortexing for 2 minutes and plating of serial dilutions onto Preston *Campylobacter* selective agar for bacterial enumeration.

2.3.5. Survival to heat stress

Briefly, 195 µL of whole milk were inoculated with 5 µL aliquots of an overnight culture of *Campylobacter* isolates (OD_{600} =0.1) in 96-well plates. The inoculated plates were then subjected to heat treatment using a thermocycler (Applied Biosystems, 2720) at 72°C for 15 and 30 seconds. Aliquots from each bacterial suspension were taken at each time interval for serial dilution and bacterial enumeration on Preston *Campylobacter* selective agar.

239

240 2.3.6. Survival to osmotic stress

241 MH broth supplemented with 0%, 2%, and 4% NaCl (wt/vol) was inoculated with 5
242 µL aliquots of an overnight culture (OD_{600} =0.1) of the *Campylobacter* isolates in
243 96-well plates. Following overnight incubation at 42°C under microaerobic condi-
244 tions, aliquots from each bacterial suspension were taken for serial dilution and bac-
245 terial enumeration on Preston *Campylobacter* selective agar.

246 2.4. Biofilm formation

247 Crystal violet staining technique was used for the quantification of biofilm formation of
248 *Campylobacter* isolates as previously described (Pascoe *et al.*, 2015). *Campylobacter* iso-
249 lates were inoculated into MH broth with OD_{600} adjusted to 1. Then, 5 µL aliquots of the
250 bacterial suspensions were used to inoculate 195 µL of liquid MH media in 96-well plates
251 (Thermo Fisher Scientific, Hudson, NH, USA). Following incubation of the plates under
252 microaerobic conditions with shaking at 42°C for 48 h in a sealed container (to prevent
253 sample evaporation), the culture media was gently removed, and the wells were washed
254 with phosphate-buffered saline. Fixation of the bacteria adhered to the wells was performed
255 by adding 200 µL of Bouin's solution (7.5 mL picric acid; 2.5 mL 40% formaldehyde; 0.5
256 mL acetic acid), incubating for 15 minutes followed by washing 3x with PBS. Plates were
257 left to air dry in an inverted position and the adhered bacteria were stained using 200 µL of a
258 0.1% (w/v) crystal violet solution for 5 minutes. Extraction of crystal violet from the ad-
259 hered bacteria was performed by adding 200 µL of an 80:20 (vol/vol) ethanol/acetone solu-
260 tion, followed by incubation for 10 minutes. The OD_{600} value of the extracted crystal violet
261 was measured using a microplate ELISA reader. These OD_{600} readings were used to quanti-
262 fy the bacterial adherence to the surface and the ability of the isolates to form biofilms. The
263 whole procedure was also repeated under aerobiosis. The isolates were classified according
264 to their OD_{600} score into strong biofilm producers (SBPs) with OD_{600} values above 0.272,
265 moderate biofilm producers (MBPs) with OD_{600} values between 0.201 and 0.272, and weak
266 biofilm producers (WBPs) with OD_{600} values below 0.201. Broth cultures with no bacterial
267 inoculation and stained using the same method were used as a negative control and the val-
268 ues obtained were subtracted for background correction. To assure the reproducibility of the
269 results, each isolate was tested for its biofilm formation in triplicate.

270

271 2.5. Statistical analysis

Statistical analysis included the comparison of different isolate groups based on their biofilm formation ability and stress resistance profile at the various time points (for aerophilic and microaerophilic growth and survival under refrigeration, freeze-thaw and heat stress) or stressors concentrations (PAA and NaCl stress) tested. Log_{10} CFU/mL values from the stress tolerance assays were compared using Wilcoxon test with the R-package *stats* v3.6.2. Plots were performed using the R-packages *ggplot2* and *ggpubr*, and statistical results were added with the *stat_compare_means* command within the ggplot function.

3. Results

3.1. High occurrence of hyper-aerotolerance among *Campylobacter* isolates

In total, 111 *Campylobacter* isolates from three different sources were screened for their aerotolerance (Figure 1A; Supplementary Figure S1A). All *Campylobacter* isolates grew well under microaerobic conditions with a steady increase in viable counts of up to 2 log_{10} CFU/mL regardless of the species or source of isolation (Figure 1B).

A high prevalence of HAT isolates was observed (63%, 70/111). Under aerobic incubation, HAT strains remained viable after a 24 h with a final mean concentration of 3.25 ±2.7 log_{10} CFU/mL, whereas 22.5% (25/111) of the isolates were AT and maintained their viability for 12 h with a final mean concentration of 2.6 ±1.4 log_{10} CFU/mL, and 14.5% (16/111) of the tested isolates were AS and lost viability before 12 h (Figure 1A).

When analyzing the aerotolerance of the isolates by their isolation source, it was observed that HAT isolates were equally recovered from the three different isolation sources, the prevalence of HAT isolates being 66.6% (38/57) of clinical samples, 62.5% (15/24) of dairy products and 56.6% (17/30) of broiler carcasses isolates (Supplementary Figure S1).

3.2. Association of high aerobic tolerance with survival under multiple stress conditions in *Campylobacter* isolates

Storage at refrigeration temperature (4°C) for 7 days instigated a significant decrease in CFU/mL in AS and AT isolates compared to HAT isolates ($p \leq 0.01$) (Figure 1D). Half of HAT isolates (50%, 35/70) exhibited an enhanced tolerance to refrigeration temperature, reaching a final concentration of 1.5 ±1 log_{10} CFU/mL after 7 days, while only two AT isolates and none of the AS strains survived at this sampling point. Similarly, both AT and AS isolates were significantly more sensitive to freeze-thaw stress than HAT isolates ($p \leq 0.001$) (Figure 1E). Thus, while 60% of HAT isolates (42/70) survived at −20 °C on day 7, reach-

ing a final mean concentration of 2.6 ±2 $\log_{10}$ CFU/mL, only three AT isolates (12%; 3/25) survived with a final mean concentration of 1.3 ±0.3 $\log_{10}$ CFU/mL, and no survivors were obtained for AS isolates.

Consistent with the data of refrigeration and freeze-thaw tolerance tests, the aerotolerance status was significantly associated with tolerance to heat stress ($p \leq 0.01$) (Figure 1F). While the 60% of HAT isolates (42/70) survived upon exposure to 70°C for 30 sec, reaching a final mean concentration of 3.2 ±2 $\log_{10}$ CFU/mL, only four AT isolates (16%; 4/25) and two AS isolates (15.5%; 2/16) survived to such a stress, reaching a final mean concentration of 2.9 ±1.7 and 1.8 ±1.5 $\log_{10}$ CFU/mL, respectively.

Finally, HAT isolates also had a significantly higher tolerance to hyperosmotic stress at 4% NaCl than AT and AS isolates ($p \leq 0.0001$) (Figure 1H). In fact, 58% (41/70) of HAT isolates survived hyperosmolarity, with a final mean concentration of 2.7 ±1.8 $\log_{10}$ CFU/mL, while only two AT isolates (8%; 2/25) were able to survive reaching a final mean concentration of 2.2 ±0.3 $\log_{10}$ CFU/mL and none of the AS isolates survived 2% or 4% NaCl exposure.

3.3. Higher tolerance of HAT and AT *Campylobacter* isolates to peracetic acid

Exposure to PAA equally reduced the viability of HAT and AT isolates by ~4.5 $\log_{10}$ CFU/mL. The initial mean concentrations of HAT and AT isolates, 7.3 ±1.1 and 7.4 ±1.2 $\log_{10}$ CFU/mL, declined after treatment to a final viable count of 2.9 ±1.8 and 2.6 ±1.5 $\log_{10}$ CFU/mL, respectively. Based on these observations, no significant difference was revealed between HAT and AT isolates, while none of the AS isolates survived upon exposure to PAA (Figure 1G).

3.4. Differences in stress tolerance between *C. jejuni* and *C. coli*

C. jejuni was significantly more tolerant to aerobic stress than *C. coli*, showing a higher survival potential at 12 h and 24 h ($p < 0.01$) (Figure 1A), despite the fact that 52% (11/21) of *C. coli* isolates were HAT, with a final mean concentration of 2.2 ±1.7 $\log_{10}$ CFU/mL after a 24 h of aerobic incubation. Yet, HAT *C. jejuni* isolates were more abundant among the collection (65.5%, 59/90) (Figure 3C), reaching a final mean concentration 3.3 ±2.6 $\log_{10}$ CFU/mL after a 24 h of aerobic incubation.

Likewise, despite the clear reduction in CFU/mL observed for both species following exposure to PAA, the majority of *C. jejuni* isolates (80%, 72/90) exhibited a significantly

higher resistance to PAA than *C. coli* ($p \leq 0.05$) (Figure 1G), reaching a final mean concentration of 2.9 ±1.8 $\log_{10}$ CFU/mL. Remarkably, none of the *C. coli* isolates survived upon exposure to 4% NaCl, while half of *C. jejuni* isolates survived with final mean concentration of 2.75 ±1.8 $\log_{10}$ CFU/mL, and such a difference between species was significant ($p \leq 0.001$) (Figure 1H).

On the contrary, no significant differences ($p > 0.05$) were detected between the two species in their tolerance to refrigeration, freeze-thaw or heat stresses (Figure 1D, E, F), with isolates from both species displaying nearly the same final mean concentrations upon exposure to 7 days of refrigeration or freezing, and 30 sec at 70ºC, *i.e.* 1.5 ±1, 2.6 ±2.1, and 3.2 ±2.15 $\log_{10}$ CFU/mL for *C. jejuni* and 1.4 ±0.45, 1.8 ±1.15, 3.2 ±1.7 $\log_{10}$ CFU/mL for *C. coli*, respectively.

3.5. Clonal population structure and its association with stress-resistance among *C. jejuni* isolates

To evaluate whether *C. jejuni* host generalist lineages might exhibit a higher potential to survive under various stressful conditions, providing them a beneficial advantage, at least in part, for transmission between multiple hosts during bacterial transmission, the population structure of *C. jejuni* was characterized using MLST and a phylogenetic tree was constructed using concatenated gene-by-gene alignments of polymorphic core genes, isolates were grouped into two distinct clusters according to their species with *C. jejuni* being more diverse than *C. coli* in regards to their CCs distributions (Figure 3A, B).

The MLST analyses revealed that the 90 *C. jejuni* isolates were classified into 15 different CCs, while 9 were not assigned to known CCs (Supplementary Table S1). *C. jejuni* isolates were classified as ``host specialist`` isolates, belonging to ST□257 ($n = 4$), ST□464 ($n = 7$), ST□353 ($n = 3$), ST□354 ($n = 3$), ST□460 ($n = 2$), ST□1034 ($n = 2$), ST□1287 ($n = 2$), ST□42 ($n = 1$), ST□573 ($n = 1$), ST□574 ($n = 1$) and ST□658 ($n = 1$) CCs, and ``host generalist`` isolates, belonging to ST□21 ($n = 36$), ST-48 ($n = 7$), ST-45 ($n = 1$) and ST-206 ($n = 10$) CCs (Sheppard *et al.*, 2014). Among the isolated *C. jejuni*, host generalist isolates were more dominant (60%, 54/90) (Figure 3B).

3.5.1. Host generalist *C. jejuni* lineages showed an enhanced capability to respond to oxidative stress

370 The majority of host generalist isolates were found to be HAT (94.5%, 51/54) and
371 survived significantly better ($p \leq 0.0001$) after 24 h of incubation under aerobic atmosphere,
372 with a final mean concentration of 3.8 ±2.1 $\log_{10}$ CFU/mL, than isolates from other lineages,
373 which barely survived the aerobic stress and showed a drastic decline in their population,
374 reaching final mean viable counts of 1.6 ±1 $\log_{10}$ CFU/mL (Figure 2A).
375
376 3.5.2. Multi-stress tolerance among host generalist *C. jejuni* lineages
377 Isolates assigned to generalist lineages showed a significantly higher tolerance to all five
378 stress conditions (*i.e.* refrigeration, freeze-thaw, heat, PAA and NaCl) than isolates of host
379 specialist lineages, which experienced a drastic reduction in survival under the stress condi-
380 tions tested (Figure 2D, E, F, G, H).
381 With regards to refrigeration and freeze-thaw stresses, the greatest decline was observed
382 among the isolates assigned to host specialist lineages on day 7, with the survival of only
383 two isolates (final mean concentration of 1.4 ±0.3 $\log_{10}$ CFU/mL) and three isolates (final
384 mean concentration of 1.13 ±0.2 $\log_{10}$ CFU/mL), respectively; whereas 59% (32/54) and
385 70% (38/54) of generalist isolates survived for 7 days under refrigeration or freeze-thaw
386 stresses, with a final mean concentration of 1.08 ±1.5 $\log_{10}$ CFU/mL and 2.1 ±2.6 $\log_{10}$
387 CFU/mL, respectively ($p \leq 0.0001$) (Figure 2D, E).
388 Similarly, the majority of isolates assigned to host generalist lineages (68.5%, 37/54)
389 survived heating for 30 sec to reach a final mean concentration of 3.2 ±2 $\log_{10}$ CFU/mL,
390 while a low percentage (16.6%, 6/36) of other lineages survived this heat stress, with a final
391 mean concentration of 2.9 ±1.7 $\log_{10}$ CFU/mL (Figure 2F). Likewise, host generalist isolates
392 efficiently survived upon exposure to PAA and NaCl, with survival rates being significantly
393 higher than those of host specialist *C. jejuni* lineages isolates ($p \leq 0.001$ and $p \leq 0.0001$, re-
394 spectively) (Figure 2G, H). All isolates assigned to host generalist CCs survived upon expo-
395 sure to 750 ppm PAA with mean viable count of 2.9 ±1.8 $\log_{10}$ CFU/mL, while 52%
396 (19/36) of the host specialist isolates were able to survive, with a mean final concentration
397 of 2.9 ±1.8 $\log_{10}$ CFU/mL (Figure 2G). Regarding NaCl stress, 74% (40/54) of the host gen-
398 eralist isolates survived the 4% NaCl exposure, with a final mean concentration of 2.7 ±1.8
399 $\log_{10}$ CFU/mL, while only two host specialist isolates (5.5%, 2/36) survived similar NaCl
400 concentration, with mean viable cell counts of 2.6 ±0.6 $\log_{10}$ CFU/mL (Figure 2H).
401
402

3.6. Exposure to aerobic stress enhances biofilm formation in *Campylobacter*

Initially, when evaluating the differences between biofilm formation potential of *C. jejuni* and *C. coli* isolates under microaerobic conditions, both species showed similar capability of biofilm formation with a mean OD_{600} of 0.4 ±0.3 and 0.24 ±0.13, respectively, whereas under aerobic conditions the biofilm formation potential was enhanced with no significance differences being observed either between *C. jejuni* and *C. coli*, with mean OD_{600} of 0.5 ±0.36 and 0.4 ±0.25, respectively ($p > 0.05$) (Figure 1C). Interestingly, under microaerobic conditions 38.8% (35/90) of *C. jejuni* isolates were classified as SBPs, while under aerobic conditions this percentage increased to reach 78.8% (71/90). On the other hand, the prevalence of SBP isolates for *C. coli* increased from a 19% (4/21) under microaerobic conditions to 71.4% (15/21) under aerobic stress (Figure 3).

In relation to the association between aerotolerance and biofilm formation potential, it was observed that *Campylobacter* displayed variable biofilm formation capabilities depending on aerotolerance status, with HAT isolates exhibiting a significantly higher adhesion capability and biofilm formation potential compared to AT and AS isolates ($p \leq 0.0001$) (Figure 1C).

Under microaerobic conditions, more than half (52.8%, 37/70) of the HAT isolates were SBPs, with a mean OD_{600} of 0.49 ±0.2, while 34.2% (24/70) were MBPs, with a mean OD_{600} of 0.23 ±0.02. Remarkably, a significant change in biofilm formation ability was observed among the majority of HAT isolates under aerobic stress, where 95.7% (67/70) of HAT isolates were SBPs, with a mean OD_{600} of 0.58 ±0.3 (Figure 3). Also, 76% (19/25) of AT isolates were found to be SBPs under aerobic stress, with a mean OD_{600} of 0.53 ±0.2 compared to a percentage of 8% (2/25) under microaerobic conditions. Interestingly, while AS isolates were all classified as WBPs under microaerobic conditions, with a mean OD_{600} of 0.14 ±0.05, an enhancement in biofilm formation potential was also observed under aerobic stress, with 56.2% (9/16) isolates being classified as MBPs (Figure 3).

3.6.1. Clonal lineages differ in terms of their biofilm forming capabilities

Biofilm formation ability was not equally distributed among *C. jejuni* lineages. When exposed to microaerophilic conditions, host generalist isolates displayed a significantly higher potential for biofilm formation (mean OD_{600} of 0.4 ±0.28) compared to isolates of other lineages (mean OD_{600} of 0.18 ±0.09) ($p \leq 0.0001$) (Figure 2C). Under favorable microaerobic conditions, 61% of the generalist isolates were SBPs, compared to only 5.5% for isolates of other lineages. Upon exposure to oxygen, *in vitro* biofilm production abilities of

437 both generalist and specialist lineages increased (Figure 2C). Still, the potential of host gen-
438 eralist isolates to form biofilms was significantly higher (mean OD_{600} of 0.54 ±0.3) than that
439 of isolates from other lineages (mean OD_{600} of 0.3 ±0.16) ($p \leq 0.0001$) (Figure 2C), with
440 98% of the generalists being classified as SBPs and none of them as WBPs, while 50% of
441 isolates from other lineages were classified as SBPs, 27.8% as MBPs, and 22.2% as WBPs.
442 Based on these results, HAT isolates that were assigned to host generalist CCs had the
443 strongest biofilm formation potential under microaerobic "favorable" conditions, which was
444 enhanced under aerobic conditions (Figure 3B, C).
445
446 4. Discussion
447 During transmission, bacteria must tolerate suboptimal conditions to successfully
448 establish an infection in the new host (Begley and Hill, 2015). *Campylobacter* are fastidious
449 organisms that are particularly sensitive to environmental stresses and that require
450 microaerophilic (5% O_2), capnophilic (10% CO_2), and thermophilic (40-42°C) settings
451 under laboratory conditions (Garénaux, 2008). It is likely that their routes of transmission to
452 humans through the environment, farm, and wild animals may interact in very complex
453 ways (Bronowski *et al*., 2014).

454 Despite the absence of many classic stress response strategies in *Campylobacter* com-
455 pared to other enteric bacteria, such as the RpoS-mediated stress resistance system, the os-
456 motic shock regulatory system BetAB and some oxidative stress response regulatory ele-
457 ments such as *oxyR* and *soxR*, and *soxS* (Murphy *et al*., 2006), *Campylobacter* has been iso-
458 lated from environmental sources where neither the atmosphere nor the temperature were
459 optimal for its survival (Chan *et al*., 2001; Trigui *et al*., 2015). *Campylobacter* has devel-
460 oped several strategies to survive in a wide range of environmental stressors outside the host
461 and/or during food processing, which likely involve the entry into a viable but non cultura-
462 ble state (Jackson *et al*., 2009), biofilm formation and lineage□specific variations in stress
463 tolerance (Pascoe *et al*., 2015; Yahara *et al*., 2017). The stress response of *C. jejuni* has been
464 previously studied and several regulatory systems have been described, including those me-
465 diating the global "SpoT-dependent stringent response", which adjusts gene expression
466 pathways to permit survival under a wide range of hostile conditions (Gaynor *et al*., 2005),
467 and the sigma factor RpoN, which plays a significant role in the resistance of *C. jejuni*
468 against osmotic stress (0.8% NaCl) and acidic pH (Hwang *et al*., 2011).

These resistance strategies can provide the means for survival and transmission of *Campylobacter* between different animal reservoirs and hosts. To maintain food quality and ensure food safety by reducing spoilage and pathogenic bacteria on food products, various intervention methods are employed, e.g. heating, modified atmosphere packaging, low storage temperatures, or use of antimicrobials and disinfectants. Nonetheless, whether the intermediate habitat (particularly from farm to fork) of *Campylobacter* plays an important role in shaping the epidemiology of this food-borne pathogen through selecting well-adapted isolates remains unclear. Therefore, this study highlights the role of cross-protection and biofilm formation on *Campylobacter* survival and considers the potential of stress-associated resistance mechanisms for selecting highly adapted *Campylobacter* isolates that can infect multiple hosts.

Despite the common perception that *Campylobacter* is sensitive to oxygen, this study revealed that large number of isolates were capable of surviving for up to 24 h even under the most hostile atmospheric conditions. With 63% of *Campylobacter* isolates, recovered from the three different isolation sources, were found to be HAT reaching final viable count of 3.25 ±2.7 $\log_{10}$ CFU/mL after 24 h of aerobic incubation. The greater prevalence of HAT isolates was observed among *C. jejuni* compared to *C. coli* isolates suggests a high capability of *Campylobacter* isolates, particularly *C. jejuni*, to tolerate oxidative stress. In agreement with this finding, several studies have reported the isolation of aerotolerant *Campylobacter* (Oh *et al*., 2015; O'Kane and Connerton, 2017). However, in the study conducted by Oh *et al*. (Oh *et al*., 2015) aerotolerance was evaluated among 70 *C. jejuni* isolates among which only 35.7% were classified as HAT, while another study reported a higher prevalence of hyper-aerotolerance among *C. coli* than among *C. jejuni* isolates (Karki *et al*., 2018), which is opposing to the results of the present study. Yet, in these previous studies no isolates from human stool and dairy products were included. Thus, to the best of our knowledge, this is the first study documenting such a prevalence of HAT *Campylobacter* isolates from three different sources.

Despite being thermophilic and ceasing its growth abruptly at temperatures below 30°C (Hazeleger *et al*., 1998), more than half of the tested HAT *Campylobacter* isolates showed physiological activity and survived for seven days under refrigeration or freeze-thaw stress. Although *Campylobacter* does not possess genes encoding cold shock proteins (Hazeleger *et al*., 1998), unlike other foodborne pathogens (Horton *et al.*, 2000), this observation suggests that some of the *Campylobacter* isolates or lineages tested may harbor other tolerance mechanisms to respond to cold shocks. This observation is in agreement with previous stud-

ies (Oh *et al*., 2018, 2019). However, these previous investigations were focused on *C. jejuni* only, unlike the current study which embraces both *C. jejuni* and *C. coli.* From another standpoint, previous studies with *C. coli* have reported its high sensitivity to freeze-thaw stress (Stead and Park, 2000), unlike the present study, where both *C. jejuni* and *C. coli* showed similar tolerance to such a stress. HAT isolates were also significantly more tolerant to heat stress than AT and AS isolates, which disagrees with a recent study (Oh *et al*., 2019) reporting that HAT isolates displayed sensitivity to heat stress and no significant association of aerotolerance with heat resistance.

Since PAA is an effective biocide used for reducing *Campylobacter* populations (Chen *et al*., 2014) and thus on examining its effect on the survival rates of the tested isolates, it was observed that both HAT and AT isolates exhibited an enhanced tolerance to PAA. A proposed explanation for such an association is that since PAA is known to decompose to H_2O_2 and acetic acid (Yuan *et al*., 1997), therefore the high tolerance of HAT and AT isolates might be attributed to the increased oxidative stress defense, as previously elucidated (Oh *et al*., 2019).

Campylobacter is known to be sensitive to hyperosmotic stress (Park, 2002). Indeed, this bacterial pathogen can be inhibited at >2% NaCl (Doyle and Roman, 1982), a food preservative commonly used to prevent the growth of foodborne pathogens (Doyle and Glass, 2010). The tolerance to hyperosmotic stress was also related to hyperaerotolerance in the present study, with HAT isolates, entirely consisting of *C. jejuni*, showed a significant high tolerance to 4% NaCl exposure. This observation suggests that oxidative stress defense systems may provide cross-protection against osmotic stress, and vice versa, which agrees with a previous study reporting that exposure to 1% NaCl moderately upregulates genes associated with oxidative stress response in *Campylobacter* (Cameron *et al*., 2012). However, no association was documented in a similar recent study, in which the level of hyperosmotolerance was variable depending on the isolate itself rather than the aerotolerance potential (Oh *et al*., 2019).

The high prevalence of HAT *Campylobacter* isolates in the current study might be a contributing factor, at least in part, to the abundance of *Campylobacter* in diverse animal, human, and environmental reservoirs in Egypt (Omara *et al.*, 2015; ElGendy *et al*., 2018). Moreover, it is apparent that the increased aerotolerance is coupled with a tolerance to temperature shifts and high osmolarity, and a possible augmented action of oxidative stress defense enzymes decomposing PAA, which will give raise to increase transmission of multi-stress tolerant phenotypes.

It has been reported that microenvironments created within biofilms permit *Campylobacter* survival for long period of time under aerobic atmosphere conditions, providing physical protection of cells from oxygen inactivation (Joshua, 2006). Indeed, in this study, biofilm formation by *Campylobacter* significantly increased under aerobic conditions, with no significant differences in biofilm formation potential between *C. jejuni* and *C. coli*, contradicting previous reports stating that *C. coli* forms less biofilms on inert surfaces than *C. jejuni* (Sulaeman *et al.*, 2010). In addition, 95.7% of HAT *Campylobacter* isolates were found to be SBPs under aerobic conditions and developed biofilms more efficiently than AT and AS isolates. Thus, signifying that *Campylobacter* can thrive in hostile environments in biofilms and highlights the role of oxidative stress as one of the signals that induce biofilm formation in *Campylobacter*, therefore contributing to its dissemination and persistence in poultry houses and slaughter facilities.

In accordance with the current study that revealed isolates from generalist lineages (CC-21, CC-45, CC- 48, CC-206) showing a substantial tolerance to temperature variation, disinfectant and high osmotic pressure, a recent genome wide association study demonstrated that some major *C. jejuni* lineages (CC-21, CC-45) possess certain genetic determinants of fitness, associated with tolerance to various pressures encountered through the poultry processing chain (Yahara *et al*., 2017). Furthermore, isolates of generalist lineages exhibited hyper-aerotolerance potential compared to isolates from other lineages. These findings are in agreement with a previous study (Oh *et al*., 2015) highlighting the enhanced aerotolerance potential of CC-21 and CC-45, which are known to be the major CCs implicated in human gastroenteritis (Nielsen *et al*., 2010; Colles and Maiden, 2012), while recently CC-48 isolates showing hyper-aerotolerance have been reported (Kiatsomphob *et al*., 2019). Nonetheless, and to the best of our knowledge, the aerotolerance potential of isolates from ST-206 CC has not been reported before.

The enhanced aerotolerance exhibited by generalist *C. jejuni* lineages in the current study might impact their potential for multi-host and foodborne transmission, which is further augmented by the high ability to form biofilms, enabling their survival outside the host and promoting their spread, as previously hypothesized (Woolhouse *et al*., 2001). Indeed, in the present study, host generalist *C. jejuni* isolates had a higher biofilm formation potential and produced more dense biofilms in oxygen-rich conditions than in oxygen-limited conditions, with 61% and 98% of generalist *C. jejuni* isolates were SBPs under microaerobic and aerobic conditions, respectively. Such an observation is consistent with a previous study show-

ing that production of biofilm increases the protection from aerobic stress in host generalist *C. jejuni* more than in other host specialist lineages (Pascoe *et al*., 2015).

In conclusion, the results of this study provide an evidence that stress-adapted *Campylobacter* lineages can thrive better under various environmental stresses than other non-adapted lineages, whereas oxidative-stress defense responses were shown to be associated with enhanced biofilm formation capabilities and with tolerance to various other stress conditions, likely due to "cross- protection" mechanisms. Our results show dominance of multi-stress resistant generalist isolates, which, together with the low biosecurity and presence of backyard farming and small-scale livestock production in Egypt (El-Tras *et al*., 2015), can provide opportunities for the multi-host spread of robust *Campylobacter* lineages. More importantly, they suggest that selective pressures encountered in hostile environments prevailing throughout food processing have shaped the epidemiology of *C. jejuni* in Egypt by selecting the transmission of highly adapted isolates, thus promoting the colonization of multiple host species by important disease□causing lineages and their transmission to humans (Sheppard *et al.*, 2009b; Yahara *et al*., 2017). Further studies will be crucial to elucidate the molecular determinants and assessing the transcriptome involved in stress or osmoregulation among HAT, AT, and AS isolates which can subsequently lead to establishing efficient measures to control the risk of *Campylobacter* in the food chain.

Abbreviation
Aero-Sensitive (AS)
Aero-Tolerant (AT)
Clonal Complexes (CCs)
Core genome Multilocus Sequence Typing (cgMLST)
Hyper-Aerotolerant (HAT)
Moderate Biofilm Producers (MBPs)
Mueller-Hinton (MH)
Multi-Locus Sequence Typing (MLST)
Optical Density at 600 nm (OD600)
Peracetic Acid (PAA)
Sequence Types (STs)
Strong Biofilm Producers (SBPs)
Weak Biofilm Producers (WBPs)

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Author Contributions
M.E., A.A.O. and S.K.S. designed the study and performed the phenotypic studies
S.F.M. wrote the paper with some additional edits from M.E and A.A.O.

J.K.C. and B.P. sequenced and assembled the genomes
S.F.M. and J.F.C. performed genomic data analysis.
J.F.C performed statistical analysis and phylogenetic tree construction with some additional contribution from A.M.
M.E. contributed to the acquisition of samples.
All authors contributed and approved the final manuscript.

Conflict of Interest
All authors declare no conflict of interest.

Footnotes
1. https://pubmlst.org/organisms/campylobacter-jejunicoli/

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Figure and Table legends

Figure 1. Survival under stress conditions and biofilm formation ability of 70 HAT, 25 AT and 16 AS *Campylobacter* isolates. (A) Behavior under aerobic stress; (B) Growth in microaerobic conditions; (C) Biofilm formation under aerobic and microaerobic conditions; Tolerance to (D) refrigeration, (E) freeze-thaw, (F) heat, (G) PAA, and (H) NaCl stresses. Statistical significance was determined using the Wilcoxon test (ns: $p > 0.05$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$).

Figure 1. Survival under stress conditions and biofilm formation ability of 90 *C. jejuni* and 21 *C. coli* isolates. (A) Behavior under aerobic stress; (B) Growth in microaerobic conditions; (C) Biofilm formation under aerobic and microaerobic conditions; Tolerance to (D) refrigeration, (E) freeze-thaw, (F) heat, (G) PAA, and (H) NaCl stresses. Statistical significance was determined using the Wilcoxon test (ns: $p > 0.05$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$).

Figure 2. Survival under stress conditions and biofilm formation ability of host generalist lineages (*n=54*) and other lineages (*n=36*) of *C. jejuni*. (A) Behavior under aerobic stress; (B) Growth in microaerobic conditions; (C) Biofilm formation under aerobic and microaerobic conditions; Tolerance to (D) refrigeration, (E) freeze-thaw, (F) heat, (G) PAA, and (H) NaCl stresses. Statistical significance was determined using the Wilcoxon test (ns: p > 0.05, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001).

Figure 3. Phylogenetic trees for (A) 21 *C. coli* isolates and (B) 90 *C. jejuni* isolates, using concatenated gene-by-gene alignments of polymorphic core genes (from cgMLST analysis) by the Neighbor-Joining method, the Jukes-Cantor substitution model and 1000 bootstrapping. A total of 525 and 320 core genome polymorphic genes were obtained for *C. coli* and *C. jejuni*, respectively. Bootstrapping values are indicated for each branch in blue text. Isolate names were substituted by their Sequence Type (ST) whenever they are known and kept for those isolates with unknown CC according to MLST analysis. Only ST-828 was found for *C. coli* isolates (indicated in the middle of the tree). (C) Summary of the available information on the isolates of *C. coli*, "*C. jejuni* generalist lineages" and "*C. jejuni* specialist lineages" (columns): aerotolerance phenotypes are shown in rows and biofilm formation ability under aerobic conditions is shown through the color code within the pie charts. The size of the piecharts is proportional to the number of isolates belonging to each category.

Figure S1: Comparison of survival rate under stress conditions and biofilm formation ability of 111 *Campylobacter* isolates based on the source of isolation. (A) Behavior under aerobic stress; (B) Growth in microaerobic conditions; (C) Biofilm formation under aerobic and microaerobic conditions; Tolerance to (D) refrigeration, (E) freeze-thaw, (F) heat, (G) PAA, and (H) NaCl stresses. Statistical significance was determined using the Wilcoxon test. (ns: $p > 0.05$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$).

Table S1: Bacterial isolates characteristics of 111 *Campylobacter* isolates and their survival rates under six stress conditions (aerobic stress, refrigeration, peracetic acid, freeze-thaw, heat, and NaCl) and biofilm formation potential under aerobic and microaerobic conditions.

905 Table S2: Whole genome sequence assembly quality features of 111 Egyptian *Campylobac-*
906 *ter* isolates.
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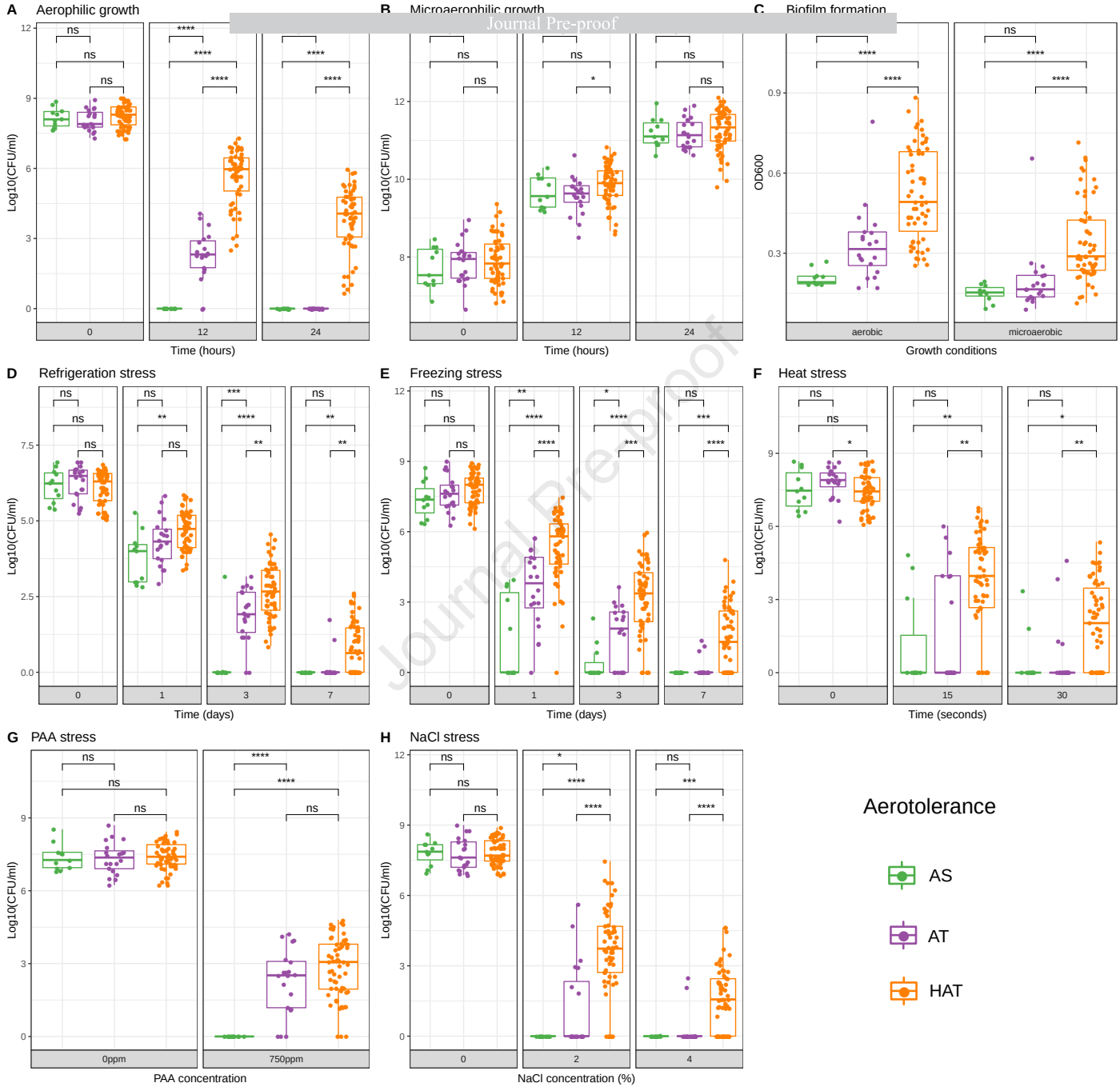

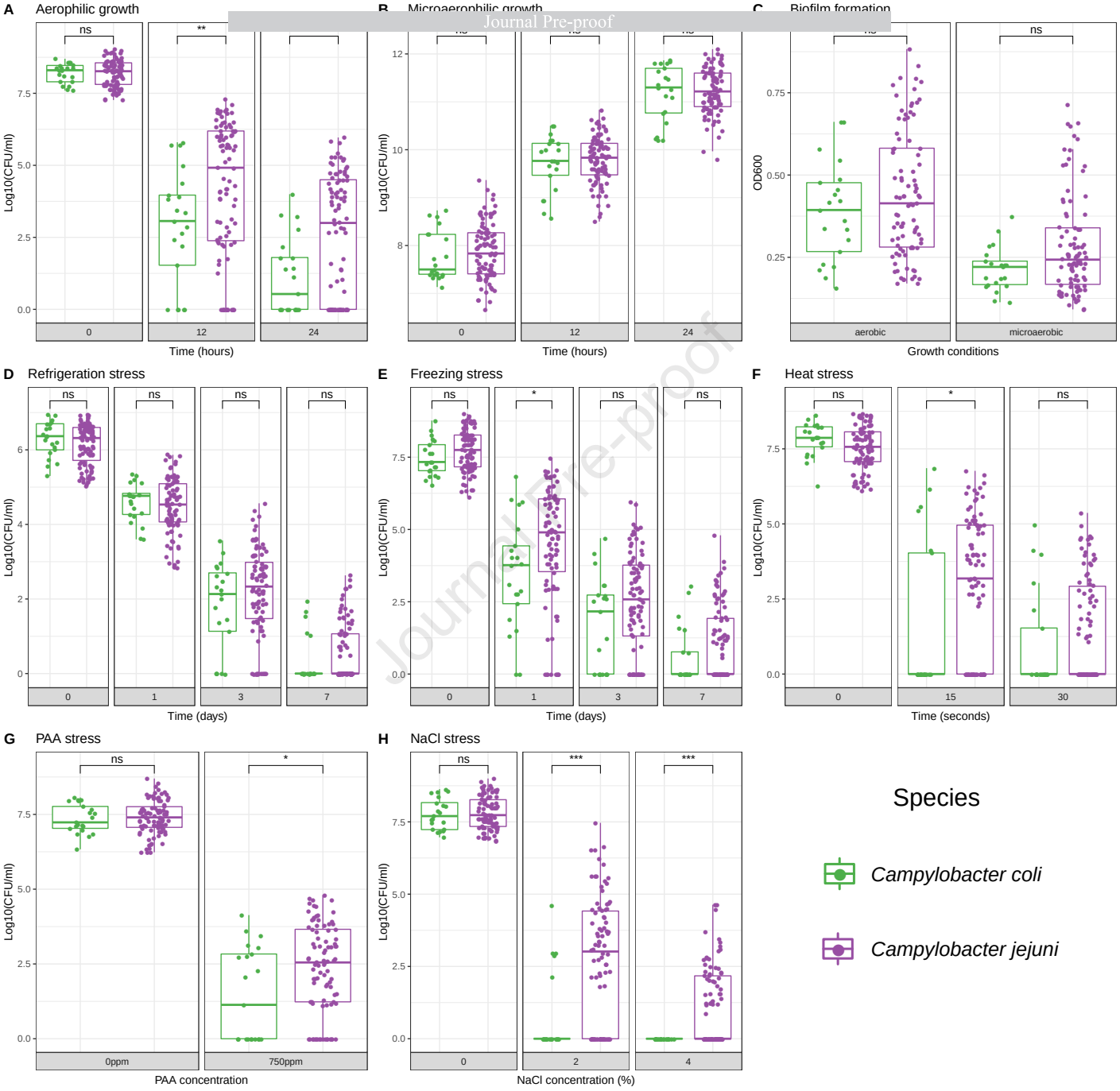

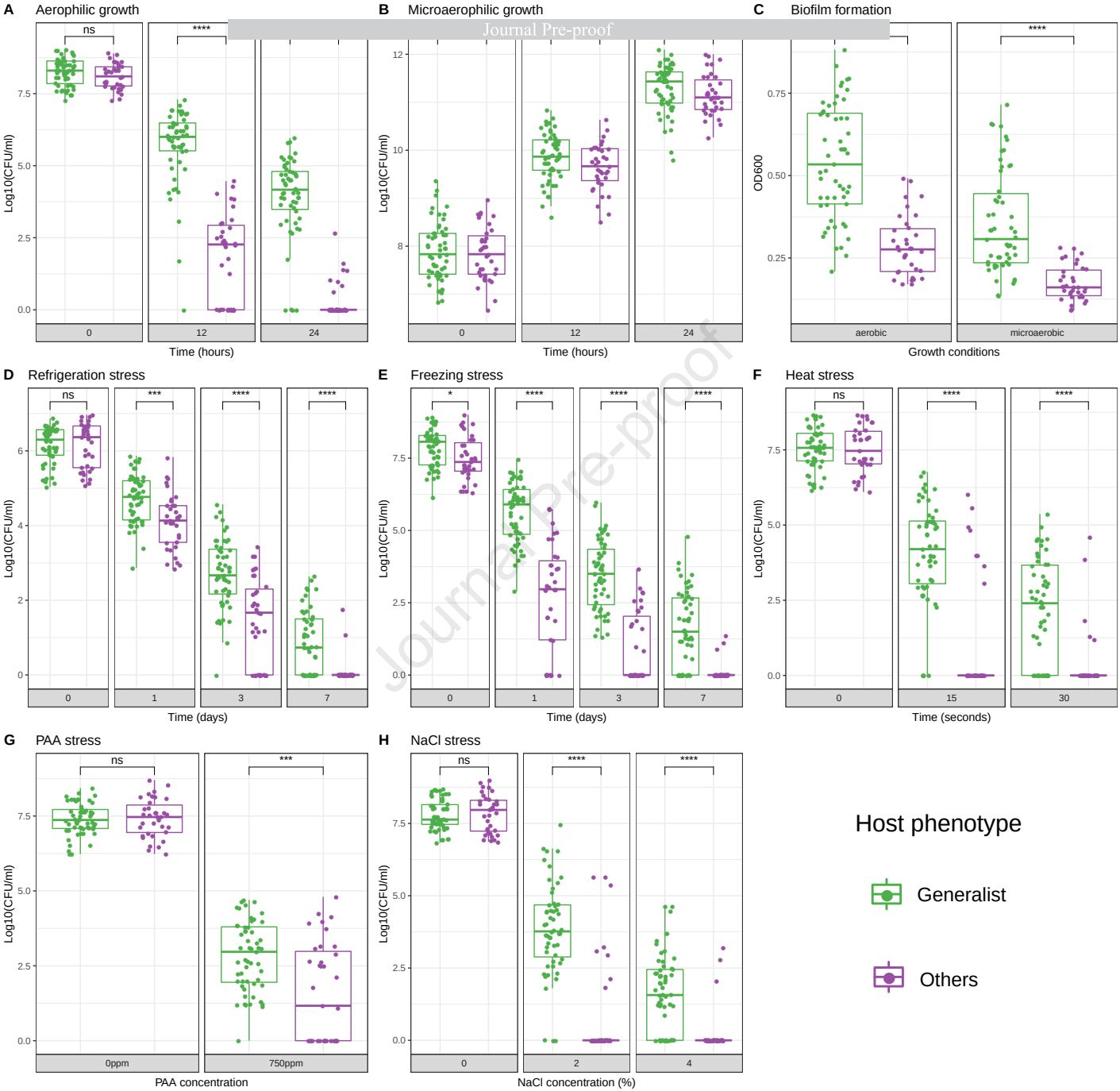

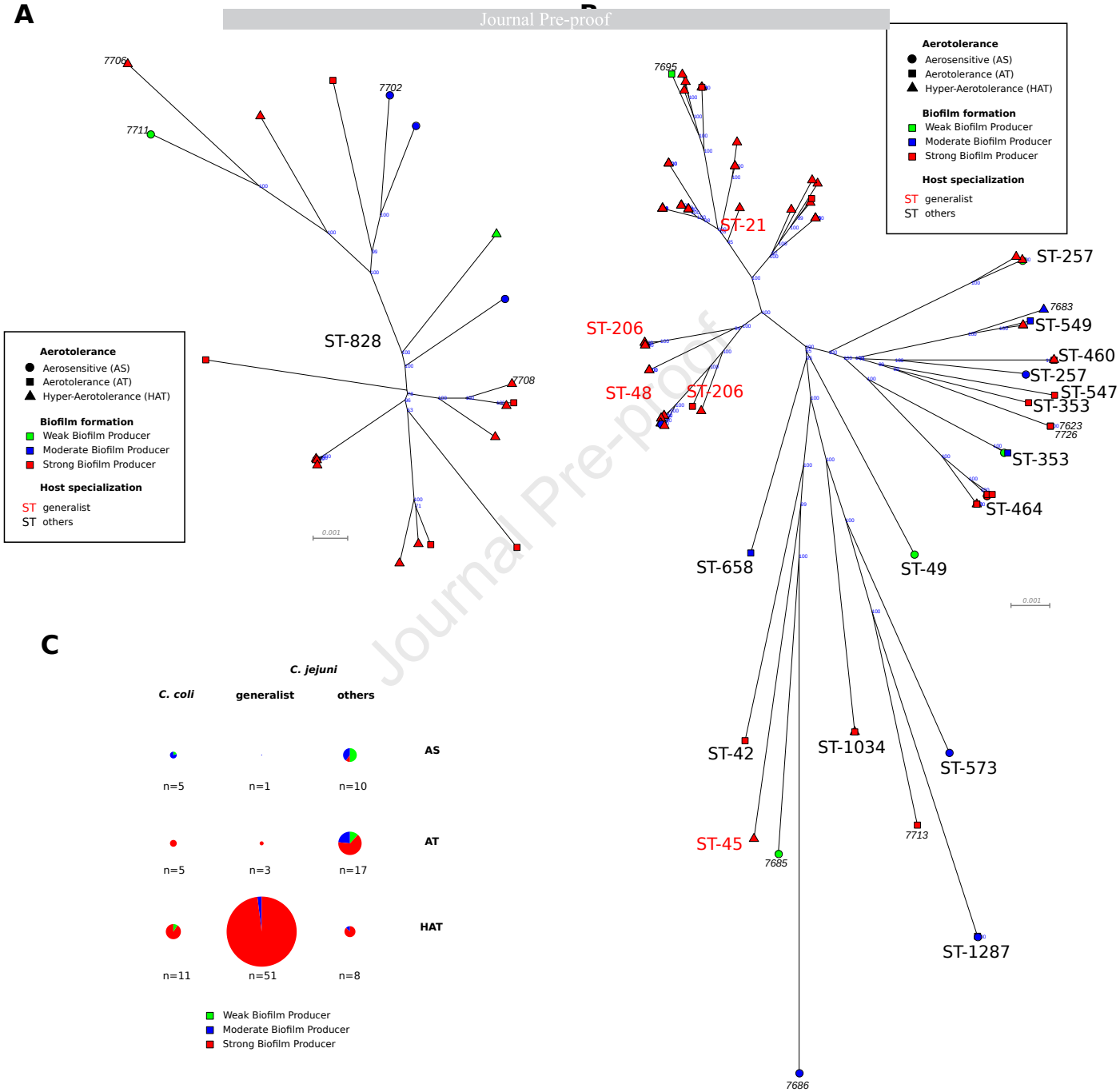

- Oxidative stress defense responses in *Campylobacter* are associated with enhanced biofilm formation capabilities
- Host generalist lineages are better equipped to withstand hostile environmental conditions favoring zoonotic transmission
- Multi-stress adapted *Campylobacter* isolates challenges efforts made to eliminate this foodborne pathogen from the food chain

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: